How do calcium channels transport calcium ions?

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Calcium channel activity is crucial for many fundamental physiological processes ranging from the heart beat to synaptic transmission. The channel-forming protein, of about 2000 amino acids, comprises four domains internally homologous to each other. Voltage-dependent Ca^{2+} channels are the most selective ion channels known. Under physiological conditions, they prefer Ca^{2+} over Na^{+} by a ratio of about 1000:1. To explain at the same time the exquisite ion selectivity and the large Ca^{2+} ion turnover rate of Ca^{2+} channels ($\approx 3x10^{6}$ ions/s), two kind models have been proposed. In one, the conduction pathway possesses two high-affinity binding sites. When two Ca^{2+} ions are bound to each site, the mutual repulsion between them speeds the exit rate for the ions, causing greater ion permeation through the pore. The second model hypothesizes the existence of a single site having a charged structure able to attract multiple, interacting ions, simultaneously. Recent studies that combine mutagenesis and electrophysiology show that the high-affinity binding site is formed by a ring of glutamate residues located in the pore forming region of the Ca^{2+} channel. As proposed in the second class of models, the results suggest that four glutamate residues, one glutamate donated by each repeat, combine to form a single high-affinity site. In this review the different conduction models for Ca^{2+} channels are discussed and confronted with structural data.

Key terms: *calcium channels, cation binding site structure, ion selectivity.*

INTRODUCTION

Voltage-dependent calcium channels control calcium entry into several types of cells, modulating their electrical activity and many metabolic responses. Voltage-dependent calcium channels are members of a superfamily of ion channels in which the fourth transmembrane segment (S4) of each domain contains several positively charged amino acid residues: the S4 superfamily (Jan & Jan, 1989, 1990). The calcium channel protein (α subunit) consists of four homologous domains in tandem, each with six (S1-S6) predicted membrane-spanning segments (Tanabe *et al*, 1987). Mutagenesis and electrophysiology have made it possible to identify the residues linking every fifth and sixth transmembrane (P region) domain as contributing to pore formation (Heinemann *et al*, 1992).

Under physiological conditions, Ca^{2+} channels transport Ca^{2+} ions efficiently and with a very high degree of selectivity over monovalent cations. According to reversal potentials measurements estimated under bi-ionic conditions, the relative permeability sequence is $Ca^{2+} > Sr^{2+} > Ba^{2+}$ for divalent

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ions and $Li^+ > Na^+ > K^+ > Cs^+$ for monovalent ions. These cations are much less permeant than the divalent ions; for example, the cardiac L-type Ca^{2+} channel shows a permeability ratio P_{Ca}/P_{Cs} of about 4200 (Hess *et al*, 1986). This selectivity is much greater than Na^+ channel selectivity for Na^+ over K^+ (P_{Na}/P_K=12) (Tsien *et al*, 1987). Surprisingly, in the absence of external Ca^{2+}, Ca^{2+} channels can carry large monovalent ion currents. Actually, the single-channel conductance of Ca^{2+} channels for Na^+ is 90 pS, more than one order of magnitude larger than the conductance for Ca^{2+} (Hess *et al*, 1986). External Ca^{2+} blocks sodium currents and the analysis of this blockade showed that the dissociation constant for the blocking reaction is about 1 μM (Almers *et al*, 1984).

The above results strongly suggest the presence of high-affinity Ca^{2+} binding sites located in the pore. However, tight binding of an ion to the site would slow Ca^{2+} conduction through the pore to values much lower than those found experimentally; there is a difficulty in reconciling high fluxes with high-affinity sites in pores (*e.g.*, Armstrong, 1975; Kostyuk *et al*, 1983). In order to solve this apparent paradox, Almers and McCleskey (1984) and Hess and Tsien (1984) proposed the existence of two high-affinity binding sites in Ca^{2+} channels that –under physiological conditions– are occupied by one or more Ca^{2+} ions. Monovalent ions in the presence of Ca^{2+} do not permeate because when Ca^{2+} occupies the sites, they are electrostatically repelled from the pore. On the other hand, repulsion between Ca^{2+} ensures the high observed single-channel fluxes. The existence of a Ca^{2+} binding site in the P-region of Ca^{2+} channels was unveiled using a series of amino acid substitutions (Kim *et al*, 1993; Yang *et al*, 1993). High-affinity Ca^{2+} binding involves four conserved glutamate residues in the P-region of domains I-IV of the α subunit of Ca^{2+} channels.

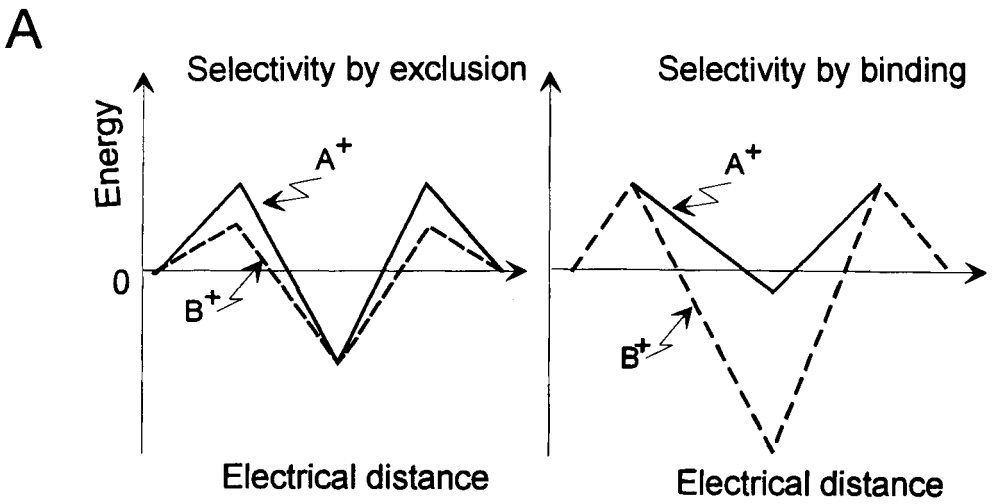
In this review, we discuss the experimental evidence that leads to the models for ion conduction in Ca^{2+} channels, as well as the molecular determinants of Ca^{2+} selectivity in Ca^{2+} channels. This evidence in Ca channels has been useful for understanding how can an ion pore show a selectivity mechanism based on high affinity ion binding sites and high ion turnover rates. These findings have been extremely useful in our understanding of how can an ion channel show an exquisite ion selectivity and a high ion turnover rate at the same time.

SELECTIVE CALCIUM PERMEATION IN CALCIUM CHANNELS IS MEDIATED BY SPECIFIC BINDING AND MULTIPLE OCCUPANCY

Selective passage of ions through an ion channel can occur by two different mechanisms: a) selection of the ion by specific binding; or b) exclusion of the less permeant ion. For several years the permeation mechanisms proposed by Bezanilla and Armstrong (1972), Armstrong (1975) and Hille (1975) were of great influence in our way of thinking of how ions were selected in ion channels. They forcefully argued in favor of a mechanism whereby the pore structure *excludes* the impermeant ion. Armstrong (1975) stated that: *"the first possibility [mechanism a)] is suitable for conferring selectivity on a carrier...but it cannot make a pore selective"*. The arguments in favor of an exclusion mechanism can be summarized with the help of Eyring energy diagrams (Fig 1A). Figure 1A considers the simplest saturable system, a channel with one site (see also Fig 1B). In the case of selectivity by exclusion, we show the hypothetical energy profiles for two ions A^+ and B^+ (solid and dashed curves, respectively). The energy profile described by the dashed curve implies that ion B^+ associated faster with the site when compared with an ion A^+ that "sees" an energy profile described by the solid line. In this case, ion B^+ excludes A^+ from the pore. Both Hille (1975) and Bezanilla and Armstrong (1972) showed that in this case the permeability ratio P_B/P_A is given by the relation (see steps in permeation described in Fig 1B)

$$P_B/P_A = k_{1B}/ k_{1A} = k_{-2B}/ k_{-2A} \qquad (1)$$

Equation (1) implies that ion selectivity depends only on the ratio of the association constants.



B

$$X_o^+ + \text{Site} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X\text{-Site} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} X_i^+ + \text{Site}$$

Fig 1. Mechanism of selectivity. **A**. Potential energy diagrams for two ions A^+ (solid line) and B^+ (dashed line) in two limiting cases of ion selectivity. Abscissae, fraction of transmembrane electric field experienced at any given point within the pore. Left hand, selectivity by exclusion. Dissociation equilibrium constants for ions A^+ and B^+ are the same, but entry rate constants are different. Ion A^+ faces a higher barrier when entering the site than ion B^+; ion A^+ is excluded. Right hand, selectivity by binding. Entry rate constants are equal, but dissociation rate constants differ. B^+ is bound selectively, but movement of ion B^+ through pore is retarded. **B.** Kinetic scheme describing ion transport in a one-site channel.

Selectivity is determined by barrier heights relative to the outside and ***not*** by well depth. Consider, on the other hand, the energy profiles given in the right-hand side of Figure 1A. In this case ion B^+ binds much tighter to the pore than ion A^+ and hence dwells longer in the pore. By deepening the well we have slowed conduction through the pore. Actually, ion B^+ in this case behaves as a blocker of the passage of ion A^+.

How can we make an ion channel selective by an affinity mechanism? Consider the energy diagram presented in Figure 2A. In this case, we drew the energy profile of the Ca^{2+} channel for Ca^{2+} (solid line) as consisting of three barriers and two binding sites (3B2S model). On the other hand, Figure 2B shows the interconnections among the four possible kinetic states of a Ca^{2+} channel with double occupancy: two Ca^{2+} can dwell in the Ca^{2+} channel pore at the same time. At low $[Ca^{2+}]$, flux is mainly through the lower cycle, as $[Ca^{2+}]$ is increased, Ca^{2+} should move through the upper cycle (*i.e.*, 0Ca $\leftrightarrow$ CaCa $\leftrightarrow$ Ca0 $\leftrightarrow$ 0Ca). At high enough $[Ca^{2+}]$ the channel is most of the time in the CaCa configuration and it is expected that channel conductance decreases with $[Ca^{2+}]$ since the conduction machinery is locked-in a non conductive configuration (Hladky, 1972; Hille & Schwarz, 1978; Finkelstein & Andersen, 1981). Notice that in the model of Figure 2A, the Ca^{2+} ions bind very tightly and the lower cycle in Figure 2B should, in principle, not produce an appreciable Ca^{2+} flux. However, when the channel is doubly occupied, ***electrostatic repulsion*** between the two divalent cations can be important,

forcing one ion to exit the channel. The magnitude of this energy of repulsion will depend on the distance separating the two ions and of the dielectric constant of the medium (see equation 2).

Is this a reasonable model for ion conduction in Ca^{2+} channels? Consider the experiment showed in Figure 3A (Almers & McCleskey, 1984; see also Hess & Tsien 1984; Fukushima & Hagiwara, 1985) which shows the fractional inward current as a function of the external $[Ca^{2+}]$. In this case, current is carried mainly by Na^+ at $[Ca^{2+}] < 10^{-5}$ M. [1] Increasing Ca^{2+} hinders the passage of Na^+, but –surprisingly– as the $[Ca^{2+}]$ becomes larger that 10^{-4} M, the current starts increasing. [2] Given that this current is carried by Ca^{2+} and that the current increases in the $[Ca^{2+}]$ range 1-10 mM, we are forced to conclude that the pore can bind at least two Ca^{2+} ions. The first calcium ion that enters to the pore blocks Na^+ currents. The $[Ca^{2+}]$ necessary to halve the Na^+ current is $7x10^{-7}$ M and corresponds to the dissociation constant, K_d, of the reaction $00 \leftrightarrow 0Ca$ (singly occupied channel). Since $\Delta G = -RTln(1/K_d)$, the well depth in the energy diagram of Figure 2A is -14.2 RT or –35.2 kJ/mol. Calcium block of Na^+ currents is voltage dependent, supporting the notion that the divalent cation must enter the pore in order to reach its binding site (Fukushima & Hagiwara, 1985). More evidence that the Ca^{2+} is in the conduction pathway of the channel is given below.

How efficiently can the singly occupied channel transport Ca^{2+}? From Figure 2, it is clear that, in the singly occupied channel, the ion flux will be determined by the rate at which ions leave the channel (*i.e.*, k_{-1}). From the state diagram showed in Figure 2B, $k_{-1}/k_1 = K_d$. Assuming that k_1 is diffusion limited and that the target has a radius of 0.3 nm, Almers and McCleskey (1984) showed that k_1 is of the order of 10^9 $M^{-1}s^{-1}$ (for a detailed discussion on these points see also Tsien *et al*, 1987). On the other hand, the measured blocking rate constant in the case of Ca^{2+} block of Li^+ currents is about $5x10^8$ $M^{-1}s^{-1}$ (Lansman *et al*, 1986; Kuo & Hess, 1993a), indicating again that the rate at which Ca^{2+} is loaded into the site is essentially diffusion limited. Hence, $k_{-1} = K_d\ k_1 \approx 7x10^{-7} \times 10^9 = 700$ ions/second. However, the observed fluxes of Ca^{2+} through single Ca^{2+} channels are much higher. For example, Rosenberg and Chen (1991) reported that in symmetrical 100 mM $CaCl_2$ the single-channel current is about 1 pA at 125 mV applied voltage. ***This current corresponds to a turnover rate of $3x10^6$ ions/s, a value that is 4200-fold larger than that estimated from the singly occupied channel.*** What is the possible origin of this difference? In doubly occupied channels, the energy of repulsion, ΔG, is given by the expression:

$$\Delta G = Ne^2z_1z_2/4\pi\varepsilon_o\varepsilon r \qquad (2)$$

where N is Avogadro's number, e is the electronic charge, z_1 and z_2 are the valence of the ions, ε_o is the polarizability of free space[3], ε is the dielectric constant, and r is the distance separating the two ions. Given a $\varepsilon = 20$, and $r = 1$ nm, equation (2) predicts an energy of repulsion between two Ca^{2+} of 28 kJ/mol or 11.3 RT. When two Ca^{2+} dwell at the same time in the pore, the energy wells are 2.9 RT (11.3 RT shallower than of wells of singly occupied channel). In the doubly occupied channel, $K_d = 41$ mM and the rate of exit increases to $6x10^7$ ions/s.[4] In spite of the assumptions implicit in the calculations, we think that the above estimations are in good

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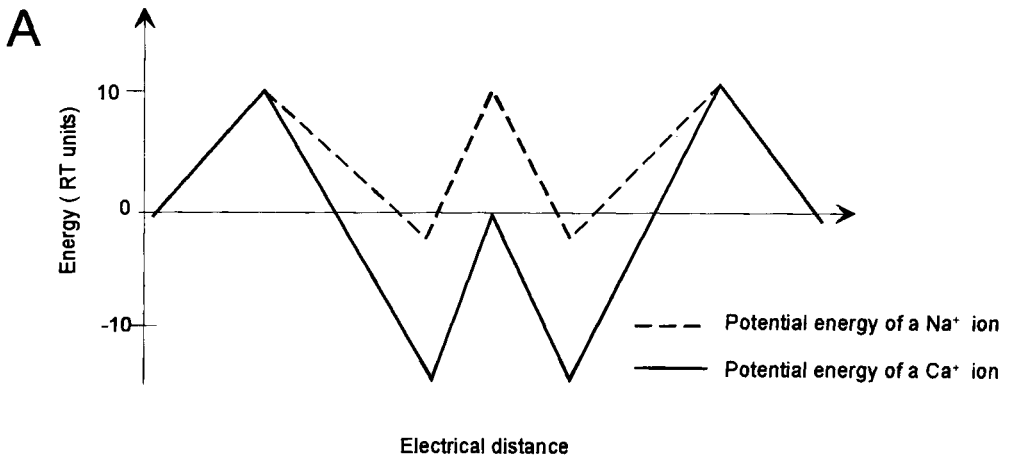
[1] It can be shown that the current at low $[Ca^{2+}]$ is carried by sodium by replacing the external Na+ by an impermeant ion like tetramethylammonium (Almers & McCleskey, 1984).

[2] It is important to note here that an appreciable amount of Ca^{2+} current is elicited at $[Ca^{2+}] \geq 1$ mM. Therefore, in the physiological $[Ca^{2+}]$ range, the channel conducts Ca^{2+} regardless of the fact that $[Na^+] = 32$ mM.

[3] A useful hint to calculate the energy in SI units is that the constant $1/4\pi\varepsilon_0 = 9 \times 10^9$ Nm^2/C^2, hence if we express e in coulombs and r in meters, ΔG is automatically given in Joule/mole.

[4] This value is obtained by taking $k_{-1}^s = 700$ ions/s $= (kT/h)exp(-\Delta G^{*s}/RT)$ for the singly occupied channel. For the case of the doubly occupied channel $\Delta G^{*d} = -\Delta G^{*s} - 11.3$ RT. Therefore, for the doubly occupied channel, $k_{-1}^d = (kT/h)exp(-(\Delta G^{*s} - 11.3\ RT)/RT) = k_{-1}^s e^{11.3} = 6 \times 10^7$ ions/s.



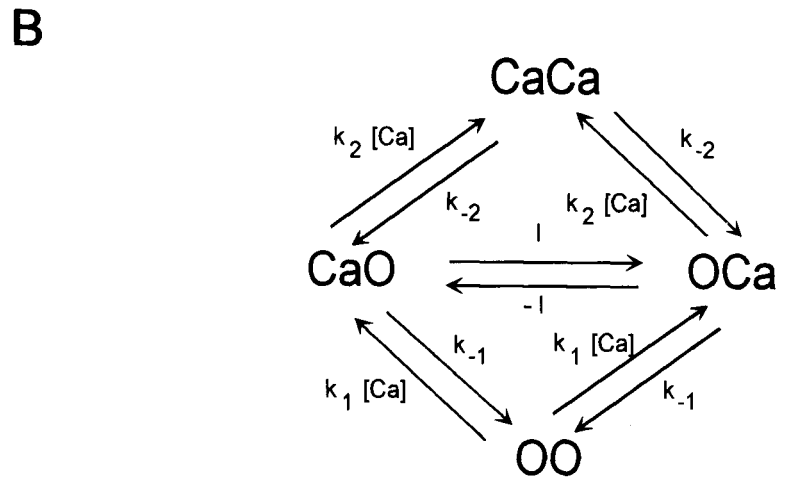


Fig 2. A. Potential energy diagrams representing steps of Na^+ and Ca^{2+} movement through three-barriers and two-sites ion channel. Ca^{2+} binds selectively to the sites. Values for energy barriers and wells for Na^+ transport are 10.0 RT and –2.0 RT, respectively. For Ca^{2+}, values for outer barriers, central barrier and wells are 10.3 RT, 0 RT, and -14.5 RT, respectively (Almers & McCleskey, 1984). **B.** Diagram showing state of occupancy of two-site channel. The channel can be occupied by more that one ion at a time, and ions only jump into vacant sites. Rate constants l and –l correspond to internal transition between sites; k_1 and k_2 describe entry rates of a Ca^{2+} ion to an empty or singly occupied pore; k_{-1} and k_{-2} correspond to exit rates of a ion. At high Ca^{2+} concentrations, Ca^{2+} moves through upper cycle; in this case the channel follows a single-vacancy model (*e.g.*, Lu & MacKinnon, 1994).

agreement with the experimental results (see below). Therefore, the results are consistent with the hypothesis that measurable Ca^{2+} flux only takes place through the upper cycle of Figure 2B. For this case, the dependence of the zero-voltage conductance, γ, on $[Ca^{2+}]$ is given by the relation (Lu & MacKinnon, 1994).

$$\gamma = (2e^2/kT)\{k_2 k_{-2} l[Ca^{2+}]/((k_2[Ca^{2+}] + 2l)(k_2[Ca^{2+}] + 2k_{-2})) \quad (3)$$

and the maximum conductance, γ_{max}, the channel can attain, is given by:

$$\gamma_{max} = (2e^2/kT)[k_{-2}l/(4\sqrt{k_{-2}l} + 2(l + k_{-2})] \quad (4)$$

Equation (3) predicts that the conductance reaches a maximum and then decreases (see above). In this case, γ_{max} (eq 4) is solely dependent on the exit rate k_{-2} and the internal transition rate l, and is

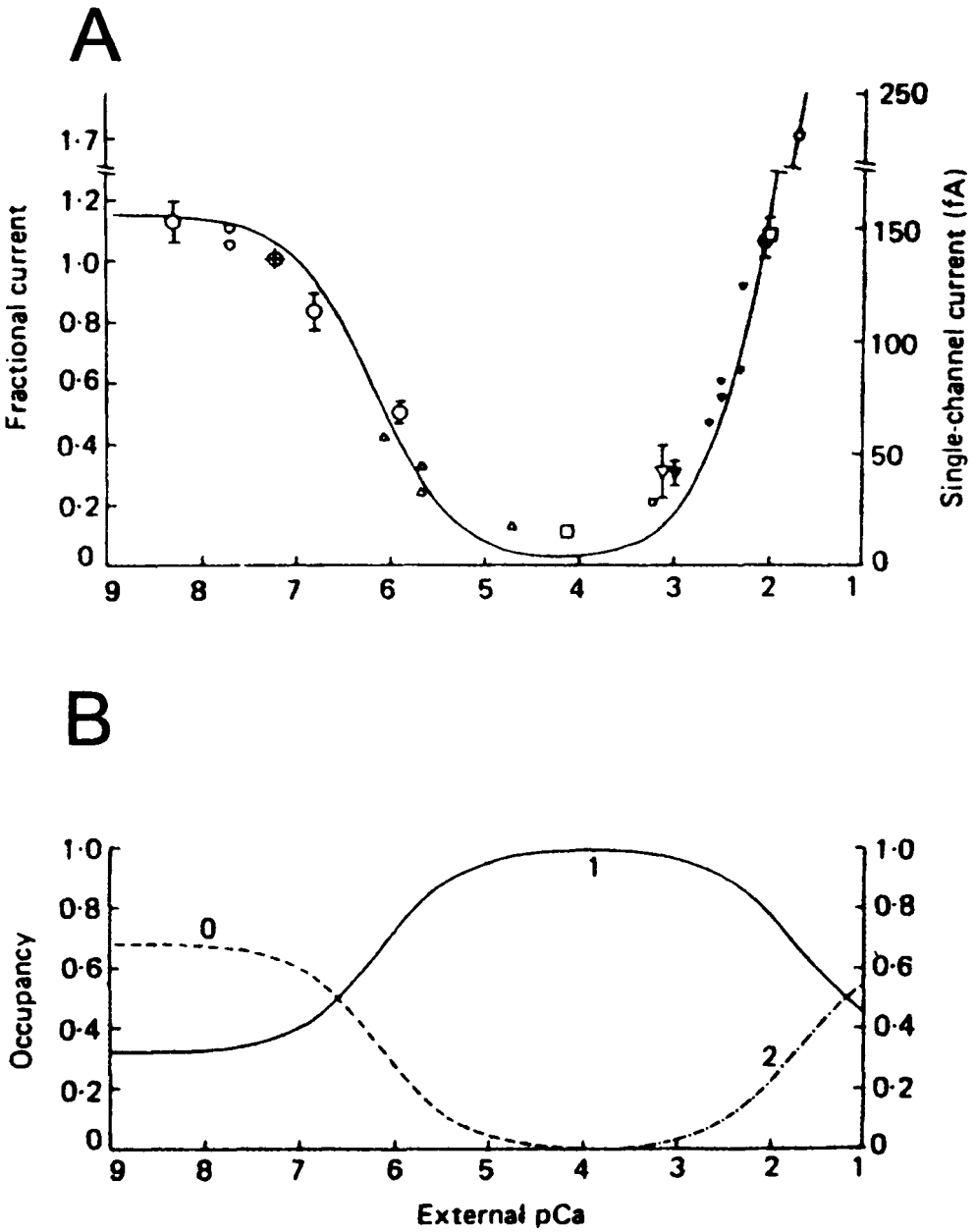


Fig 3. A. Ion currents through calcium channels as a function of pCa (= $-\log[Ca^{2+}]_o$). Data obtained from macroscopic currents recordings in skeletal muscle. Open symbols plotted as fractional current referred to value obtained at pCa = 7.2; filled symbols plotted as fraction of value at pCa = 2. Solid curve, values of unitary currents calculated with energy profiles showed in Fig 2A. Parameters used for Ca^{2+} were: $[Ca^{2+}]_i$ = 0, height of outer barriers 10.3 RT, central barrier 0, depths of wells -14.5 RT. Parameters for Na^+ were: $[Na^+]_i = [Na^+]_o$ = 32 mM, all barriers 10.0 RT, all wells -2.0 RT. Membrane potential of -20 mV and ion repulsion energy of 11.9 RT were assumed. At pCa > 4, inward current is carried by Na^+. Ca^{2+} only carries significant current at $[Ca^{2+}] > 10^{-4}$. **B.** Occupancy of channel by Ca^{2+} as a function of external pCa for three-barriers and two-sites model. Diagram shows fraction of pores occupied by zero (dashed curve), one (solid curve), or two (dashes and dots) ions. Na^+ flux is blocked when pore becomes occupied by one Ca^{2+} ion. Increased current above millimolar concentrations parallels increased probability of double occupancy by Ca^{2+} ions. Single occupancy of pore explains block of Na^+ flux with K_d of 1 μM and double occupancy explains saturation of Ca^{2+} flux with K_d of 15 mM. (Modified from Almers & McCleskey, 1984).

independent of the entry rate. Experimentally, the curve γ vs. calcium concentration is well described by a rectangular hyperbola of the type $\gamma = \gamma_{max} / (1 + K_d/[Ca^{2+}])$ with a K_d = 15 mM (Almers & McCleskey, 1984). This value is close to our naive calculation (41 mM) that considers double occupancy and electrostatic repulsion. However, in the range of $[Ca^{2+}]$ tested no decrease in channel conductance is apparent. This is not surprising since the $[Ca^{2+}]$ at which the γ_{max} is reached depends heavily on the entry rate k_2 (see figure 2B and Lu & MacKinnon, 1994). It is possible that in the case of Ca^{2+} channels the value of k_2 determines that γ_{max} is reached at $[Ca^{2+}]$ not attainable experimentally.

More evidence for multiple ion occupancy in Ca^{2+} channels: Ca^{2+} sites are located near the external mouth of the pore.

A major distinguishing feature that has favored multi-ion occupancy in Ca^{2+} channels is the *"anomalous mole fraction effect (AMFE)"* between Ca^{2+} and Ba^{2+}. The AMFE describes the observation that in the presence of a solution containing a mixture of Ca^{2+} and Ba^{2+} where $[Ca^{2+}] + [Ba^{2+}]$ = constant, the channel conductance is smaller compared with the conductance in pure solutions containing either ion (Hess & Tsien, 1984; Almers & McClesky, 1984). The AMFE was not found in cardiac L-type Ca^{2+} channels at single channel level (Yue & Marban, 1990), and the validity of the AMFE as an indicator of multiple occupancy has been severely questioned by Eisenman and Horn (1983). However, more recent evidence has supported a multi-ion pore for Ca^{2+} channels.

Kuo and Hess (1993b) demonstrated that no matter from which side the blocking Ca^{2+} ion comes, the exit rates are always the same at the same potential in the same direction of Li^+ current. This finding can be explained if there is only one high-affinity binding site or one **set** of sites in the pore, so no matter whether Ca^{2+} comes from inside or outside, it always goes to the same site. Set can be conceived as a cluster of sites separated by insignificant barriers. They also demonstrated that raising the external $[Li^+]$ (when Li^+ current is outward) or internal $[Li^+]$ (when Li^+ current is inward) can ***decrease*** the exit rates of the blocking Ca^{2+}. Figure 4A shows the case when current is outward and the external $[Li^+]$ was raised. These results provide evidence that the channel can hold at the same time a Li^+ and a Ca^{2+} ion and that the occupancy of the channel by Li^+ makes Ca^{2+} dwell for longer times in the pore. This effect, found first in Ca^{2+}-activated K^+ channels, has been termed "lock-in" effect (Neyton & Miller, 1988). The "lock-in" effect is easily explained by postulating the existence of one or more low-affinity Li^+ binding sites. In other words, Ca^{2+} ions are flanked by Li^+ that hinders (locks) the exit of the divalent cation (Fig 4A). The Ca^{2+} channel can hold several ions in its pore. Moreover, when the external $[Li^+]$ is increased and the current is inward, the exit rate of a blocking ion is increased and becomes negligible when the $[Li^+]$ approximates zero. This is the "enhancement" effect (Fig 4B) that was also found previously in Ca^{2+}-activated K^+ channels (Cecchi *et al*, 1987) and shows the ***necessity of having at the same time a Li^+ and Ca^{2+} ion in order for the latter to exit the channel.***

As stated above, if an ion must enter the pore to reach its blocking site, the reaction between the blocking ion and the channel will be voltage-dependent. In this case, we say that the ion travels an "electrical distance" before finding its site. Kuo and Hess (1993b) found that for external barium[5] there is a very short electrical distance between the external mouth of the pore and the high-affinity binding site (see also Chow, 1991). Considering this short distance, the existence of a low-affinity site

[5] In Kuo and Hess (1993a, b) experiments, Ba^{2+} instead of Ca^{2+} was used as the permeant cation. The single channel conductance for Ba^{2+} is larger than for Ca^{2+} and the slow channel inactivation present when Ca^{2+} is used as the permeant cation is not present when Ba^{2+} is used as the external cation.

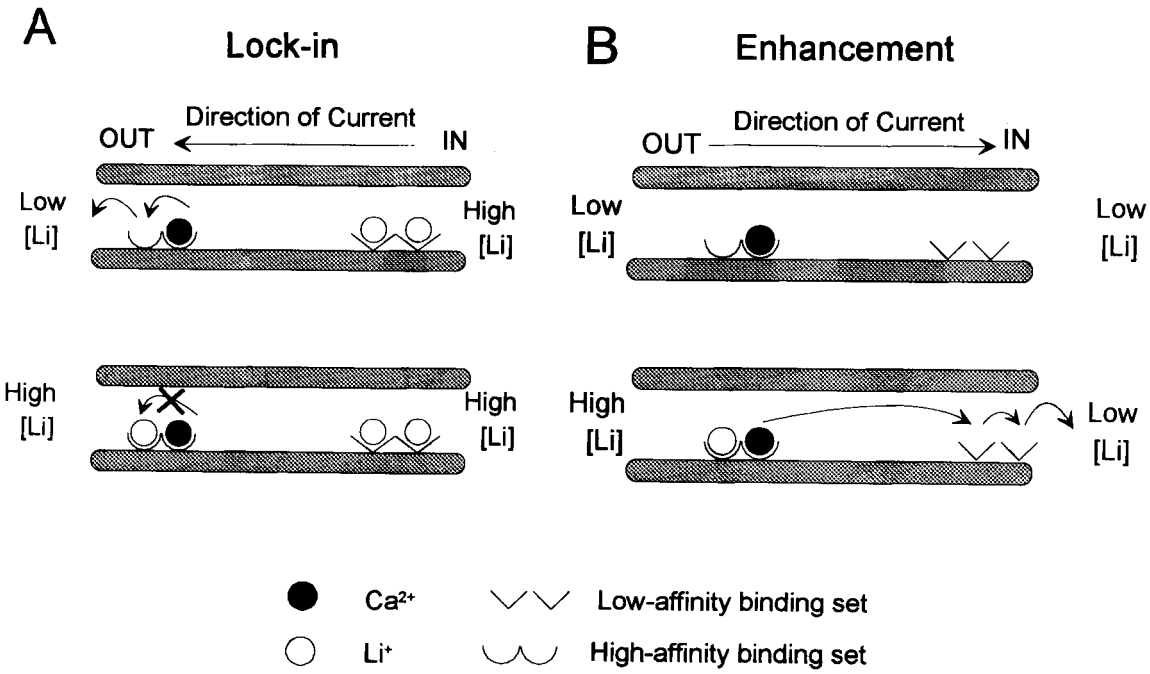


Fig 4. Schematic diagrams describing ion "lock-in" and ion "enhancement" effect. Kuo and Hess (1993b) proposed that Ca^{2+} channel is a multi-ion pore that contains two sets of binding sites, one composed of high-affinity sites and located near external pore mouth, and other composed of low-affinity sites and placed internally. **A.** When Li^+ current is outward and external [Li^+] is increased, Ca^{2+} ion can be locked in the site. **B.** When Li^+ current is inward and external [Li^+] is increased, exit of a Ca^{2+} ion is enhanced by presence of a Li^+ ion in external high-affinity site. (Modified from Kuo & Hess, 1993b).

between them is unlikely. Hence, it is necessary that the high-affinity site accept at least a Li^+ and a Ba^{2+} at the same time, to explain the "enhancement" and the "lock-in" effect caused by variation of *external* [Li^+]. To determine how many sites are in the high-affinity set, additional experiments were necessary. Competition experiments between a permeant ion such as Ba^{2+} and a blocker such as Cd^{2+} put an upper limit to the number of sites. If we assume the existence of only two sites, then the competition reaction between Ba^{2+} and Cd^{2+} is given by the following kinetic scheme:

$$\underset{\text{CdBa}}{} \overset{K_{Cd}}{\leftrightarrow} \underset{\text{OBa}}{} \overset{K_{Ba}}{\leftrightarrow} \underset{\text{BaBa}}{} \qquad \text{(R-1)}$$

where K_{Cd} and K_{Ba} are the dissociation constant for Cd^{2+} from the CdBa-channel and for Ba^{2+} from the BaBa-channel complexes, respectively. In this case the CdBa-channel complex is a non-conductive species (the channel is blocked) and the probability of finding a blocked channel, P_{CdBa}, is given by the following expression (from Vergara & Latorre, 1983):

$$P_{CdBa} = 1/\{1+K_{Cd}(1+([Ba]/K_{Ba}))(1/[Cd])\} \qquad (5)$$

Kuo and Hess (1993b) found that the apparent K_d of external Ba^{+2} to prevent the entry of an external blocking ion such as Cd^{2+} (K_{Ba} in eq 5) is similar to the Kd of pushing off a Ba^{2+} ion, required for permeation (Ba^{2+} concentration dependence of conductance, *i.e.*, $\gamma = \gamma_{max}/(1 + K_d/[Ba])$. In other words, and according to scheme R-1, both sites have to be occupied by Ba^{2+}, either to prevent entry of Cd^{2+} or to push off a Ba^{2+}. However, if there are three sites, the equality between the K_{Ba} obtained from competition and conductance measurements is unlikely. In this case, to prevent Cd^{2+} entry, it is necessary for one Ba^{2+} to take the first site and the other the third site (*i.e.*, BaOBa, the configuration with lowest free energy). On other hand, to push off a Ba^{2+} ion, the ions have to be in adjacent sites, where ion-ion interactions are

maximized. The probability that BaOBa and OBaBa configurations have the same free energy is very low, so the Kuo and Hess (1993b) results limit the number of high-affinity binding sites to no more than two.

However, the above idea is in contradiction with experiments of "lock-in" and "enhancement" effects. The effect of increasing external Li^+ over blockade by Ca^{2+} when Li^+ current is outward (lock-in), can be represented by the following kinetic scheme, where two high-affinity sites are considered:

Direction of Li^+ current ←

$$\text{Out} \quad OO \xrightarrow{K_{off}} OCa \underset{}{\overset{k_d^{Li}}{\leftrightarrows}} LiCa \quad \text{In}$$

where K_d^{Li} is the dissociation constant for Li^+-induced "lock-in" effect; the state OCa represents the blocked channel and the state LiCa represents the channel blocked with the Ca^{2+} "locked-in" the site. On the other hand, the effect of increasing external Li^+ over the Ca^{2+} block of Li^+ inward currents ("enhancement" effect) can be represented by:

Direction of Li^+ current →

$$\text{Out} \quad OCa \overset{K_d^{Li}}{\leftrightarrows} LiCa \xrightarrow{k_{off}} LiO \quad \text{In}$$

where K_d^{Li} is the dissociation constant for Li^+-induced "enhancement" effect; in this case, the LiCa complex does not contain a locked Ca^{2+} ion, because the direction of the Li^+ current is inward.

From these kinetic schemes we conclude that the same value for the dissociation constant for the LiCa complex is expected in both cases. However, this is not obtained in practice: the K_d for "lock-in" effect is in the millimolar range. In contrast, the value obtained for the "enhancement" effect is in the molar range. Considering these results, the model with only two high-affinity sites is unlikely. In order to explain this paradox, Kuo and Hess (1993b) advanced the following statement: *"The point raised...essentially argues against any 'fixed ionic site' concept about the organization of the high-affinity set. Actually all the findings can be explained successfully by a simple model of the high-affinity set which contains no discrete ionic site"*. In their view, the "sites" are very near and separated by a negligible barrier. The arrangement of ligand groups allows Li^+ to bind in different configurations than can have different K_d^{Li+} (Fig 5C, D). This view of the high-affinity set is perfectly compatible with the picture of the sites we have at present based on the pore structure (Fig 8C).

A one site model.

A hypothetical single site model for the Ca^{2+} channel was proposed by Armstrong and Neyton (1992). In this model, the site is viewed with a net charge of -2 electronic charges (for example a two carboxyl site, Fig 6A). There will be an attraction energy between this site and the divalent cation given by eq (2), where r = site radius + cation radius. For example, if the r_{site} = 0.132 nm and r_{cation} = 0.1 nm, it takes 47.5 kT for a Ca^{2+} to leave the site.[6] Therefore, the site will never be empty and the energy cost of a second Ca^{2+} approaching to the site is zero since the complex site-divalent cation is neutral. Conduction in this case occurs by exchange or, in the Hodgkin and Keynes (1955)[7] terminology, by a knock-on mechanism. A divalent cation that acquires sufficient thermal energy to approach the site has a chance of "knocking-out" the cation already present with no energy cost. Armstrong and Neyton (1992) challenge vacancy models (*e.g.*, Fig 2) by stating that *"for a charged site conduction cannot occur by a vacancy mechanism, because there is never a vacancy (strict knock-on)"*.

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[6] Equation 2 gives the energy per mol, taking $\varepsilon = 20$, it can be reduced to $^*\Delta G = 2.8\ RT\ z_1z_2/r$, where r is the distance between centers and it is expressed in nm. To obtain energy per ion, equation 2 can be modified to: $\Delta G = 2{,}8\ kT\ z_1z_2/r$.

[7] We must appreciate the fact that Hodgkin and Keynes (1955) provided the first and the strongest evidence for single filing in a pore. Measuring the unidirectional flux ratio, they showed that in the squid K^+ channel at least 3 K^+ ions can dwell simultaneously within the conduction system.

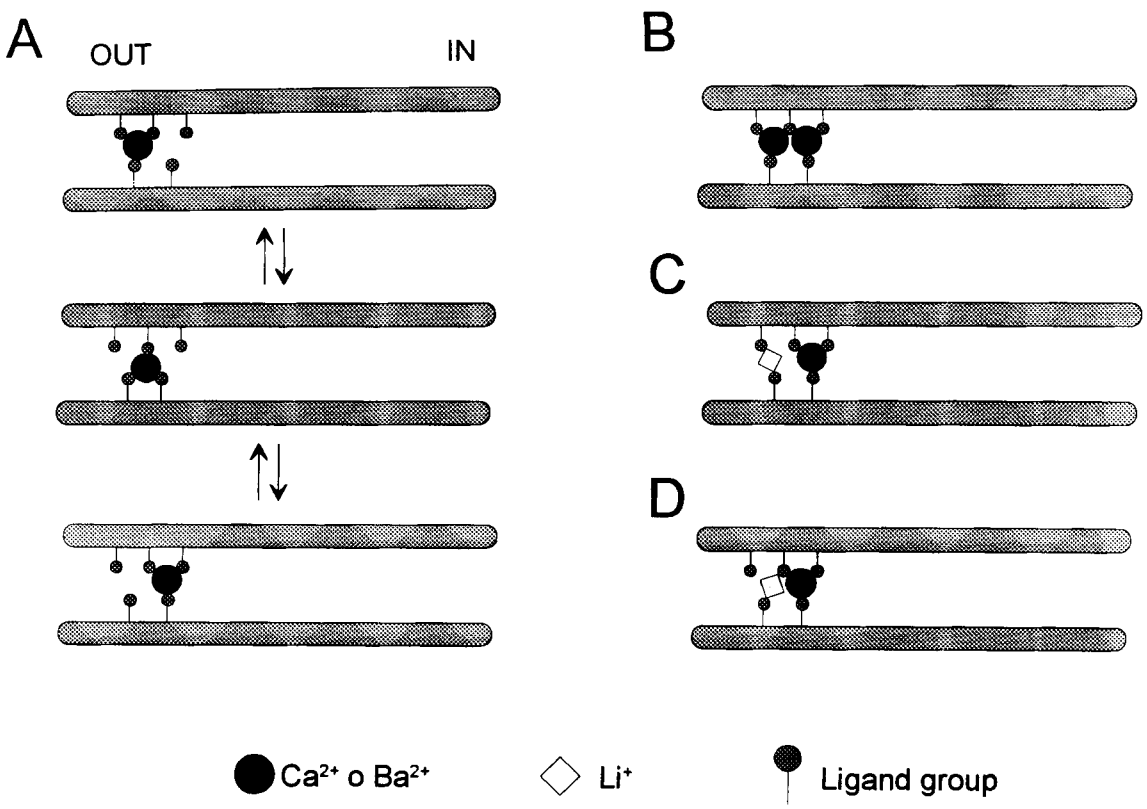


Fig 5. Schematic diagrams of high-affinity set in Ca^{2+} channel, showing it as an array of ligand groups and not as fixed or discrete sites. **A.** If the set is singly occupied, then Ba^{2+} or Ca^{2+} ions can have different positions inside the set. Energy barriers between these locations are negligible. **B.** If the set is doubly occupied by divalent ions, they can no longer move easily and are limited to a certain configuration. **C.** If the channel is doubly occupied by one Li^+ and one Ca^{2+}, the Li^+ ion can be bound in different positions. In a position far away from Ca^{2+}, the monovalent ion can prevent the outward movement of Ca^{2+} in the set ("lock-in" effect in outward monovalent currents) with a K_d in the millimolar range. **D.** In another configuration, Li^+ ion can be bound close to Ca^{2+} ion and increase inward exit of Ca^{2+} ("enhancement" effect). Apparent K_d for this position is very high (molar range). (Modified from Kuo & Hess, 1993b).

During conduction, the site alternates between double and single occupancy. To explain Ca^{2+}-current saturation, special arrangements near the site are required, where the energy of the doubly occupied must be lower than that for the singly occupied channel. Figure 6A shows a singly and a doubly occupied site, in the latter, the cations are separated by a gap. If the gap is increased, the repulsion between cations will be weaker than the attraction between a cation and the site, so the doubly occupied site energy will be lower than that of the singly occupied one! As shown in Figure 6B, another kind of arrangement can be considered. Here, in a doubly occupied site, the repulsion between cations is higher than the attraction by the site. In this case, it is the repulsion that facilitates the exit of an ion. The Armstrong and Neyton (1992) model can account for the blocking of monovalent ion flux by Ca^{2+} (Fig 6C) and the AMFE.

THE MOLECULAR NATURE OF THE HIGH-AFFINITY BINDING SITE

Figure 7A shows that the Ca^{2+} channel α subunit consists of four repeats each containing six putative transmembrane domains (S1-S6). This structure is shared by Na^+ channels, and Guy and Seetharamulu (1986) predicted that the hairpin loop between domains S5 and S6 (P-region) forms part of the pore lining. In Figure 7B we show an alignment of the amino acid sequence in the regions encompassing the P regions of the four repeats of Na^+ and Ca^{2+} channels. In the

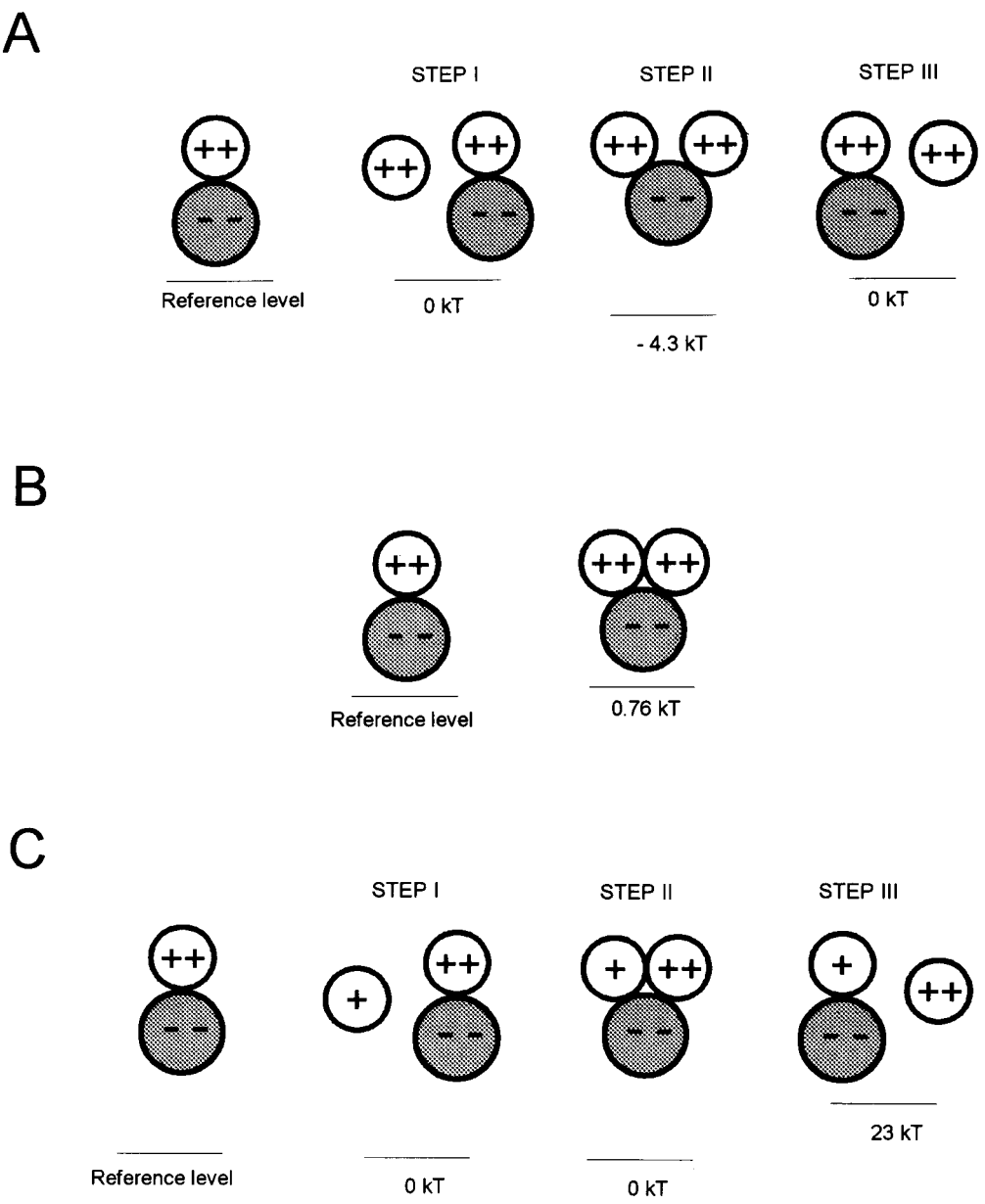
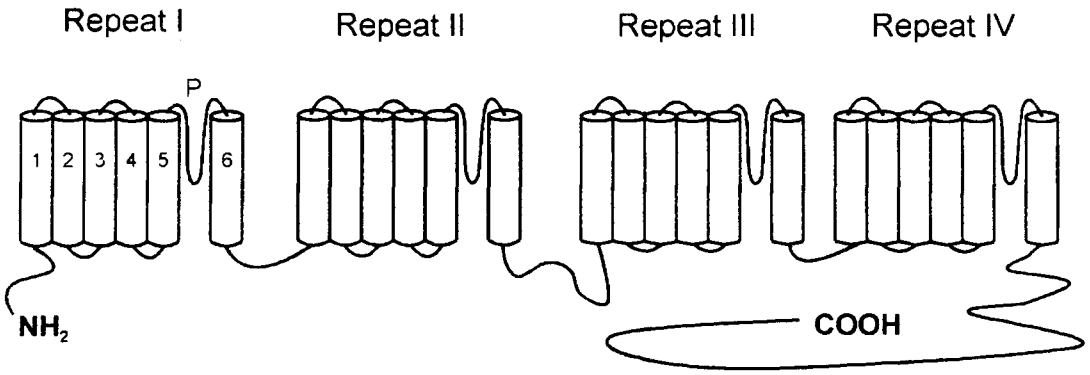


Fig 6. One site model for permeation through Ca^{2+} channels. **A.** Divalent ion conduction. During conduction, pore alternates between single- and double-occupancy states. Single occupancy state is taken as reference level. If a single divalent cation (open circle) is bound to a site with a charge of –2e (filled circle), approach of a second ion does not require energy because complex site-ion is neutral (step I). Withdrawal of second ion also does not require energy because cation is withdrawn from a neutral complex (Step III). Energy of singly occupied complex is higher than that of doubly occupied complex, when the two cations are separated by a gap. This can be explained because the attraction to the site ($-11\ kT/(r_{ion} + r_{site})$) is stronger than mutual repulsion ($11\ kT/(2r_{ion}+gap)$). For example, when r_{ion} = 0.1 nm, r_{site} = 0.132 nm and gap = 0.7 nm, doubly occupied site energy is lower by 4.3 kT than that of singly occupied site, giving value for apparent K_d of 14 mM. **B.** Array of divalent ions in the site is critical for permeation. When cations are adjoining (gap = 0), energy of this double occupancy state is higher by 0.76 kT than that of single occupancy state. **C.** Inhibition of monovalent ion current by Ca^{2+}. Scheme shows steps required for replacement of a divalent cation in the site by a monovalent ion. Approach of a monovalent ion to the site occupied by a divalent ion does not require energy, because the site is neutral. Step III would be very unlikely because divalent cation leaves from a complex that has a charge of –1e (a step that would require 23 kT of energy). Thus, a low concentration of Ca^{2+} would lead to almost exclusive occupation of sites by Ca^{2+} and very low monovalent conduction. Number under each step, change in ion-ion energy in kT (see equation 2). (Modified from Neyton & Armstrong, 1992).

A



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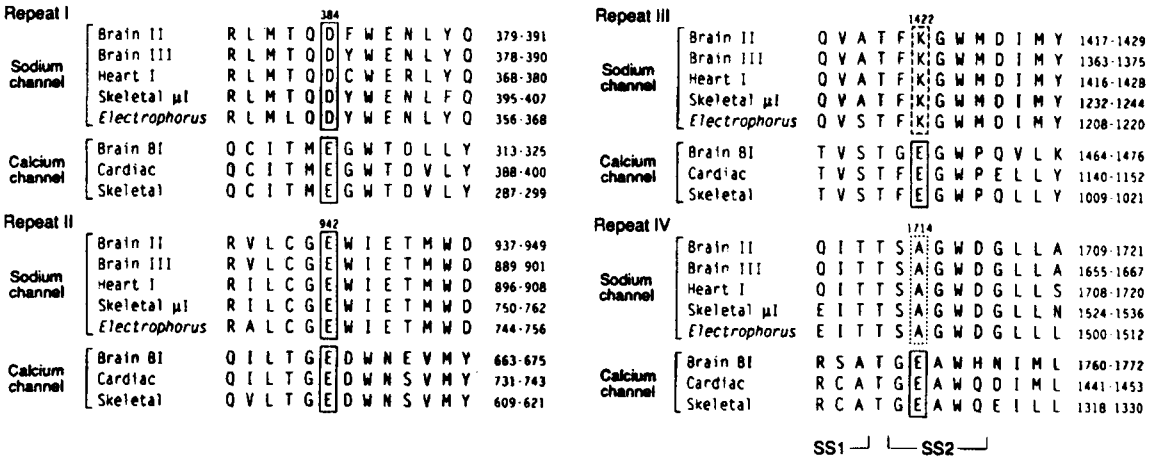

Fig 7. A. Schematic model of proposed transmembrane topology of a Ca^{2+} channel. The four repeats of Ca^{2+} channel are displayed linearly with six putative transmembrane α-helices (S5-S6) in each repeat. Regions between transmembrane segments S5 and S6 of four repeats are placed within membrane (P-region or SS1-SS2 region). **B.** Alignment of amino acid sequences in region encompassing SS1-SS2 regions of the four repeats of different Na^+ and Ca^{2+} channels. Numbers of amino acid residues shown on right side. Positions of SS1 and SS2 segments are indicated at bottom. Cluster that contains residues identified as determinants of ion selectivity is boxed (negatively charged residues, solid line; positively charged residues, dashed line; uncharged residues, dotted lines). (Modified from Heinemann *et al*, 1992).

four repeats, Ca^{2+} channels have negatively charged amino acid residues (glutamates) in equivalent positions. Na^+ channels, on the other hand, have negative charges in only two repeats, and the others contain a lysine (positively charged) and an alanine (hydrophobic). Replacing in the Na^+ channel protein the lysine in position 1422 in repeat III and the alanine in position 1714 in repeat IV by glutamates altered dramatically the sodium channel selectivity properties (Heinemann *et al*, 1992). In

particular, sodium channels lost the high selectivity over K^+, the monovalent ion currents were blocked by low [Ca^{2+}], and when the [Ca^{2+}] concentration was increased the channels begun to conduct Ca^{2+} exactly as Ca^{2+} channels do (Fig 3). ***Replacement of the lysine 1422 and alanine 1714 in Na^+ channels by glutamate conferred to sodium channels Ca^{2+} channel ion selectivity properties.*** These results were one of the first clues that these sites in both Ca^{2+} and Na^+ channels form part of the selectivity filter of these channels.

The importance of the glutamate residues in the repeats of L-type cardiac Ca^{2+} channels (Fig 8A) in determining the conduction properties of these channels is illustrated in Figure 8B (Yang *et al*, 1993; also see Kim *et al*, 1993; Mikala *et al*, 1993; Tang *et al*, 1993; Yatani *et al*, 1994; Ellinor *et al*, 1995; Bahinski *et al*, 1997). Point mutations in the individual repeats [E → Q (glycine; a neutral amino acid)] decrease the ability of Ca^{2+} to block Li^+ currents. However, the mutations affected the block of Li^+ currents unequally. The order of the [Ca^{2+}] necessary to halve the Li^+ current (IC_{50}) is EIIIQ > EIIQ > EIVQ > EIQ > wild type (Fig 8B). This finding indicates that although the glutamate residues are positioned equivalently in the aligned sequence (Fig 8A), there are functional differences between them. In Ca^{2+} channels, glutamates are not arranged forming a symmetrical ring of amino acid residues (Fig 8C). As pointed out by Yang *et al* (1993) and Kim *et al* (1993), the non-equivalence of Ca^{2+} channel glutamates might arise from asymmetry in their α-carbon atoms or under the influence of residues located in their neighborhood. We note here that in the double mutant EIQEIVQ the block is reduced by about 100-fold compared with the wild type channel. This result showed that changes in binding energy are not additive since an additive effect predicts only a 14-fold change.[8] We conclude that individual mutations do *not* contribute equally to Ca^{2+} binding.

Ellinor *et al* (1995) extended Yang *et al* (1993) results to include mutations of the four glutamates to either glutamines or alanines. The replacement of the four negatively charged amino acid residues in the Ca^{2+} channel weakened the affinity for Ca^{2+} by more than 1000-fold. This result clearly precludes the existence of well-separated second binding sites in another region of the pore. Furthermore, Ellinor *et al* (1995) went further and tested the possible arrangement of the four glutamates that could give rise to strong interaction with Ca^{2+} by effects of pairwise replacements of glutamates. We can envision that glutamates form a single high-affinity site as proposed by Armstrong and Neyton (1992) or that they form two sites close together (Kuo & Hess, 1993a, 1993b). In this regard, a specific model was proposed by Varadi *et al* (1995): EII and EIV give origin to an outer site and that EI and EIII contribute to the formation of an inner site. To test these possibilities, glutamates were replaced two at a time by alanines in all six possible combinations (Ellinor *et al*, 1995). All double mutants tested showed appreciable Ba^{2+} current and the [Ca^{2+}] necessary to halve the Li^+ current was increased from about 1 μM to 100-500 μM. These results do not support a model in which the glutamates are divided up into two well-defined binding sites; otherwise, at least one of the double mutants tested channels would be expected to show high-affinity Ca^{2+} block of monovalent ion currents but *not* divalent ion currents. These results also make the original multisite model proposed by Almers and McCleskey (1984) and Hess and Tsien (1984) untenable. In Ca^{2+} channels all four glutamate residues intervene in the high-affinity binding of the divalent cation in a manner that closely resembles the models proposed by Armstrong and Neyton (1992) and Kuo and Hess (1993b).

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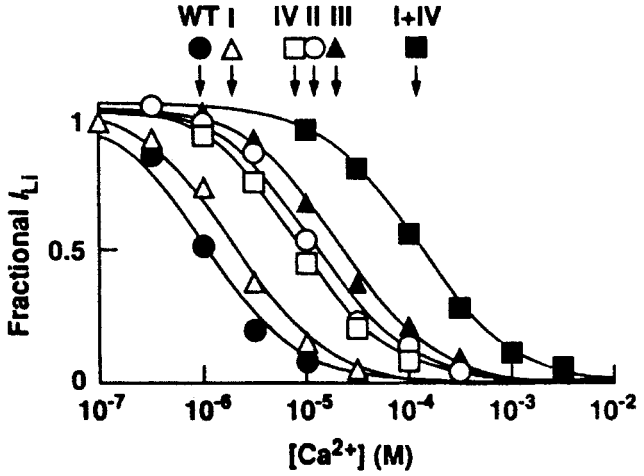
[8] IC_{50} for Li^+ block for the EIQ mutant is 1.85-fold larger and for the EIVQ mutant 7.7-fold larger than the wild type. Since $\Delta G = -RT\ln K$, energy additivity is given by the relation $K1xK2 = \exp-[(\Delta G1 + \Delta G2)/RT]$, *i.e.*, 7.7 x 1.85 = 14.

A

Repeat I	V	F	Q	C	I	T	M	**E**	G	W	T	D	386-397
Repeat II	V	F	Q	I	L	T	G	**E**	D	W	N	S	729-740
Repeat III	L	F	T	V	S	T	F	**E**	G	W	P	E	1138-1149
Repeat IV	L	F	R	C	A	T	G	**E**	A	W	Q	D	1439-1450

B



C

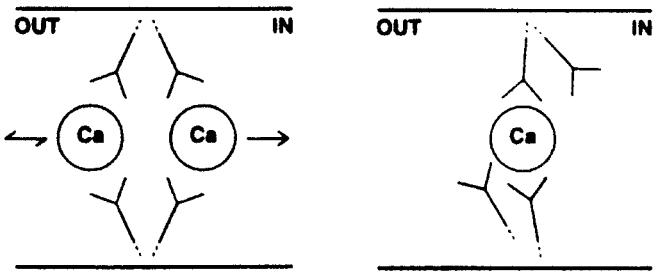


Fig 8. A. Alignment of amino acid sequences in putative pore-lining (P) regions of the four repeats of cardiac L-type Ca^{2+} channel. Residues conserved among all known voltage-gated channels are indicated in boldface. Glutamate residues replaced in site-directed mutagenesis studies are boxed. Residues numbers are shown on right. **B.** Dose-response relationships of Ca^{2+} block of Li^+ currents. Currents recorded from *Xenopus* oocytes injected with complementary RNA encoding wild type or mutant channels. Averaged fractional peak currents were plotted as a function of external Ca^{2+} concentrations. At the top, repeats with glutamate residues substituted by glutamine. IC_{50} values were 0.97 for WT and 1.85, 10.7, 18.6, 7.7 μM for mutant channels with single substitutions (glutamate(E) → glutamine(Q)) in repeats I, II, III or IV, and 104 μM for double mutant. These values are indicated by downward arrows at the top. **C.** Schematic diagrams of the four glutamate carboxylates (represented by forks), within permeation pathway of Ca^{2+} channel. An asymmetrical pore is proposed to explain unequal contribution of the four glutamates to high-affinity binding site. Diagram on left shows that the four glutamates can contribute to binding of two Ca^{2+} ions simultaneously. (Modified from Ellinor *et al*, 1993).

If the glutamates conform the only cation-binding region located in the P-region, they must be able to interact with multiple divalent cations simultaneously as demanded by the experimental results (Almers & McCleskey, 1984; Hess & Tsien, 1984; Yue & Marban, 1990; Kuo & Hess, 1993a, 1993b). In other words, independently of the model of conduction considered (*e.g.*, vacancy or knock-models), multi-ion occupancy is necessary to account for the large Ca^{2+} flux through Ca^{2+} channels. Lansman *et al* (1986) demonstrated that increasing the [Ba^{2+}] concentration not only decreases the association rate constant for Cd^{2+} block, but also increases the off-rate. This is a clear evidence that the channels can hold a Ba^{2+} and a Cd^{2+} ion simultaneously. Recent studies show that point mutations of the glutamate residues altered both Cd^{2+} block and the ability of Ba^{2+} to displace Cd^{2+} from the pore (Ellinor *et al*, 1993). It is inescapable, therefore, that the set of glutamates present in the Ca^{2+} channel can support interactions with more that one divalent cation.

CONCLUDING REMARKS

To explain the large Ca^{2+} ion turnover rate and the high selectivity of Ca^{2+} channels, several models have been proposed. All models agree that the multiple ion occupancy of the channel is necessary to account for the ion conduction properties. Ion-ion interactions are essential for decreasing the affinity of Ca^{2+} for the ion binding site. A decrease in affinity promotes an increase in the exit rate and, hence, an increase in the maximum conductance the channel can attain. This in turn implies that the turnover rate is larger in doubly than in single-occupied channels.

The electrophysiological experiments that describe ion permeation through calcium channels can be accounted for equally well by two kinds of models. Some models propose the existence of two or more high-affinity binding sites for Ca^{2+} in the conduction pathway and explain permeation on the basis of a vacancy mechanism. The other models propose a single high affinity site (described as a single site or as a single region without discrete sites) that can bind more than one ion simultaneously. In this case strict "knock-on" models for permeation are more appropriate. Recently, structural studies have resolved the controversy between these two kinds of models. Studies using mutagenesis and electrophysiology show the existence of only one high-affinity site for divalent ions. This site is formed by four glutamate residues localized within the conduction pathway.

ACKNOWLEDGMENTS

This work was supported by grant FNI 197-739, *Cátedra Presidencial* and a group of Chilean companies (CODELCO, CMPC, CGE, Minera Escondida, NOVAGAS, Business Design Ass and XEROX Chile). Gonzalo Ugarte was supported by a CONICYT fellowship, FONDECYT grant # 297006 and Postgraduate Department-University of Chile # PG/034/97.

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